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(54) Title: A METHOD FOR SEPARATING NON–PROTEINACEOUS SUBSTANCES FROM PROTEINACEOUS SUBSTANCES FOR SUBSEQUENT PROCESSING

(57) Abstract

The present invention is directed to a simple but efficient method for separating non–proteinaceous substances, such as drugs and nucleic acids from proteinaceous substances for subsequent monitoring and evaluation. The non–proteinaceous substances are captured by an environmentally sensitive solid carrier under physiological conditions and released under non–physiological conditions with a solvent, which is compatible with or used in subsequent steps. The solid carriers are provided in the form of membranes, sheets, sticks, plates, test tubes, microplates or as beads or granules attached to a further solid support. The surface of said carriers are covered with capturing residues, which are sensitive to changes in the environmental conditions, e.g. pH or ionic strength. Said residues are responsible for binding and release of drugs or nucleic acids and allows their easy and rapid separation from proteins. Test kits including said solid carriers as well as their applications are also disclosed.

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A METHOD FOR SEPARATING NON-PROTEINACEOUS SUBSTANCES FROM PROTEINACEOUS SUBSTANCES FOR SUBSEQUENT PROCESSING

The Background of the Invention

The Technical Field of the Invention

The present invention is directed to a method for performing a simple separation of non-proteinaceous substances from proteinaceous substances in biological fluid samples for subsequent monitoring and/or evaluation. The invention is also directed to a solid carrier composed of an inert porous material carrying residues, which are sensitive to environmental changes and under physiological conditions bounds non-proteinaceous substances and under non-physiological conditions releases said substances in a solvent or diluent used in the subsequent monitoring or evaluation system. Preparation of solid carriers, test kits and applications are also described.

The Technical Background

Drug monitoring is an important stage in the development of new drug molecules. Today, there is also an increased interest in monitoring therapeutic drug concentrations to evaluate the success of medication and for clinical drug monitoring to determine therapeutic drug concentrations. An even more increased interest in follow up studies, such as uptake, metabolic degradation and release of drugs can be expected in the future. Analyses of narcotics, poisons etc. are also more frequently required and the need of routine analyses for such substances can be expected to increase as well.

The most complicated, time consuming and expensive step in monitoring and evaluation of drugs, drug-like substances and drug-derivatives is the separation, isolation and purification

of drugs in inert form from proteins and other biological substances or compounds in biological samples, especially body fluids.

The most commonly used methods in clinical laboratories today require organic solvents and/or commercial materials, including stationary phases, which are immobilized on the supporting particles, such as gels, silica based materials, organic polymer based materials, e.g. alumina, silica, but also polymers, such as ethylvinylbenzene-divinylbenzene copolymer as well as compounds which are capable of forming bounds with the drug to be monitored. In reversed phase systems, the mobile phase is significantly more polar than the stationary phases. Said materials are used in combination with equipments for automatic extraction of drugs from other sample components. The destruction and disposal of solvents may cause environmental problems and risks. The extraction methods are generally time consuming, the recovery is not always sufficiently complete and/or exact. Elaborate laboratory facilities and equipment as well as highly qualified trained personnel is required.

The preparation and use of polymers covered with biological substances and/or provided with reactive groups have been described in literature and patent publications. They have generally been developed for isolation and demonstrations of proteinaceous substances and nucleic acids, etc., but so far their use for separating different types of substances, such as drugs and nucleic acid in an inert form and in a simple and rapid manner, which facilitates rapid analyses as well as other applications has not been suggested.

Polymers have been used in diagnostic methods to provide membranes for assays based on e.g. immunochemical methods and for doping tests. In these cases one or more biologically active substance is usually bound to the surface of the polymer. Often the biological material used to prepare the

membrane is capable of binding only one desired specific substance. This means that a separate membrane must be developed for each substance or drug to be monitored and evaluated. The system is an acceptable procedure for routine testing, if monitoring of one specific substance is desired, but in situations, where the substance to be isolated is not known or several types of drugs or drug-like substances or their metabolites should be monitored or evaluated simultaneously, a battery of separating devices are required. This is not an acceptable arrangement, because it is not cost-effective to use several membranes for one sample.In such cases a simple routine isolation with subsequent or simultaneous monitoring is not possible and conventional laboratory methods, which are more time-consuming, environmentally unfriendly as discussed above must be used. The used solvents, even if removed, often disturb the monitoring and evaluation methods. For example, when chromatographic methods are used, the solvent front is broad, which in turn disturbs the analysis and prolongs the performance of the test.

In immunological methods the non-specific binding of proteinaceous materials causes many problems and reduces the specificity of the test and reduces the possibilities of simultaneous immunoassays of several drugs. So far, no simple and rapid method for removing non-desired proteinaceous material from the sample has been developed.

As discussed above, there is an increased demand for rapid, efficient, simple, cost-effective and time-saving routine methods or systems for isolating, separating and purifying non-proteinaceous substances in an inert form from proteinaceous substances present in biological samples to enable routine screening of one or more substances individually or simultaneously in large amounts of biological fluid samples of different origin and for different purposes.

In the present invention the problem has been solved by constructing a solid carrier made of an inert porous, preferably polymeric material in the form of sheets, membranes, sticks, test tubes, microplates as well as granules and beads attached to a further solid support. The solid carrier comprises an inert backbone covered with residues, which are sensitive to environmental conditions, e.g. charged, functional groups, preferably a polymer forming substance with said properties. Said charged residues are in physiological, i.e. approximately neutral conditions capable of capturing other substances with relatively small molecules, such as drugs, drug-like substances and drug-derivatives and releasing said substances by changing the environmental conditions, such as pH and ionic strength. One preferred embodiment of such a carrier is a poly(acrylic acid) grafted poly(vinylidene fluoride)membrane. Another preferred embodiment of the invention is a vinylpyridine grafted poly(vinylidene fluoride) solid carrier. Said carrier is manufactured in form of a membrane, sheet, granules, test tube, microplate or as granules and beads, which might be attached to further supports.

Thus, the objectives of the present invention is not only to improve and simplify isolation, separation and purification procedures, it also improves the efficiency of the subsequent monitoring and evaluation procedures.

In immunological methods, the non-specific binding can be significantly reduced by using the method of the present invention. This provides possibilities for detection of several drugs simultaneously by immunochemical assays.

The Summary of the Invention

The characteristic features of the present invention are as defined in the claims.

The present invention is related to a method for separating non-proteinaceous substances, such as drugs and/or nucleic acids from proteinaceous substances in biological fluid samples for subsequent monitoring and evaluation. In the method of the invention a biological fluid sample is contacted under physiological conditions with a solid carrier, the surface of which is covered with a substance, preferably a polymer forming substance carrying a multitude of capturing residues. The capturing residues are sensitive to environmental conditions, such as pH and ionic strength. Under physiological conditions the capturing residues are capable of capturing non-proteinaceous substances, such as drugs and nucleic acids, but essentially none of the proteinaceous substances present in biological samples.

The solid carrier carrying the captured non-proteinaceous substances on the capturing residues is removed after a suitable time and subjected to one or more optional washing steps.

The captured non-proteinaceous substances are released from the carrier by contacting the solid carrier with a releasing solution under non-physiological environmental conditions.

The releasing solution is a solvent or diluent, which is compatible with and/or used in the subsequent method for monitoring or evaluating the captured substance.

The invention also provides a test kit for separation of non-proteinaceous substances, such as drugs and/or nucleic acids from proteinaceous substances in a biological fluid sample for subsequent monitoring and evaluation. The test kit comprises, in addition to one or more of the solid carrier described above, in a packaged combination optional washing solutions and at least one releasing solution, as well as auxiliary equipments and instructions for use.

The Brief Description of the Drawings

Fig.1 shows the reaction mechanism when grafting acrylic acid onto polyvinylidene fluoride (PVDF) membranes. In stage (1) the PVDF membrane is irradiated with electrons and in stage (2) the irradiated membrane is reacted with acrylic acid (A).

Fig. 2 is a schematic overview of one specific embodiment of the method according to the invention performed with a membrane.

Fig. 3 is a schematic overview of another specific embodiment of the method according to the invention performed in a test tube.

Fig. 4 shows the analyses with HPLC of a mixture comprising 7 drugs directly (A) and after separation on a membrane (B).

Fig. 5 shows a human sample containing two drugs analyzed with HPLC. In (A) a sample preparation made with a C18 extraction column (conventional solvent extraction) and in (B) a preparation made with a PVDF-PAA membrane is shown. The solvent front is clearly smaller in B and the time needed for separation is shorter.

Fig. 6 shows the drug release by changing the ionic strength.

The Detailed Description of the Invention

Definitions

In the description that follows, a number of terms used in pharmaceutical industry and pharmacology in relation to drug monitoring as well as in polymer chemistry and diagnostics are extensively utilized as set forth in e.g. Clarke's isolation and identification of drugs. Ed. Moffat, A.C., Jackson, J.V., Moss, M.S. and Widdop, B. The Pharmaceutical Press, London,

1991. In order to provide a clear and consistent understanding of the specification and claims, including the scope to be given such terms, the following definitions are provided.

The term "solid carrier" as used in the present invention refers to inert polymer backbones, which can be pressed or plasticized, extruded, molded or otherwise manufactured in such a form, which facilitates easy handling. The solid carrier is prepared of a porous and essentially inert polymeric material. The term "porous, inert polymeric material" is preferably a plasticized network, scaffold or backbone of a polymer manufactured in the form of a membrane, sheet, stick, bead, granule, test tube, plate or microplate. It is essential that the plastic, polymer backbone is covered with a substance, preferably a polymer forming substance, which can be provided with a multitude of functional groups, which are charged in physiological conditions and are capable of binding said non-proteinaceous substances, but essentially none of the proteinaceous substances present in the biological samples and act as "capturing residues".

The environmentally sensitive capturing residues of the solid carrier are selected from a group consisting of acrylic acid, poly(acrylic acid), vinylpyridine, or polyvinylpyridine, which are grafted on an inert polymeric substance. The most preferred embodiment of the solid carrier is a poly(acrylic acid) grafted polyvinylidene fluoride membrane.

The need of a hydrophobic backbone is not absolute. It seems as if beads of poly(acrylic acid) as such could be used as a solid carrier.

The term "solid support" as used in the present invention refers to inert polymer backbones, which can be pressed or plasticized, extruded, molded or otherwise manufactured in such a form, which facilitates easy handling. The term "solid support" in this connection is used to indicate that a "solid

carrier" as defined above, especially a bead or granule is further attached to a further solid carrier. In other words the term "solid support" means that the charged residues are provided on a solid carrier which is attached to another solid carrier. For example solid beads or granules consisting of poly(acrylic acid) can be used as "solid supports" according to the invention.

The term "capturing residue" means a functional group, which is sensitive to changes in the environmental conditions, such as pH and ionic strength. Usually the capturing residue means a functional group, which carries a charge under physiological conditions. Such groups or residues are for example carboxyl groups, amino groups or other chemical moieties including pyridine or pyrimidine, which can be attached to the solid carrier by chemical and/or physical means. Several methods of grafting by chemical means or irradiation are described in the Patent US 5,547,575. Conventional chemical methods including polymerizing can be used as well. The charge of "the capturing residues" defined above is selected so that it has a charge, or a group which has a charge, which is opposite to that of substance to be captured from the biological fluid sample. The charged functional groups are usually neutralized when the environmental conditions are changed to a non-physiological range. However, it is not a prerequisite that the "capturing residue" has a charged which is opposite to the "captured substance". The "solid carrier" also capture non-proteinaceous substances, which are not charged. However, it is a prerequisite that the "capturing residues" do not capture substances, which have the same charge as the capturing residue nor proteinaceous substances.

The term "physiological conditions" in this connection means a pH in the range of 5-7.5 or a ionic strength of approximately 0.15, i.e. in the range of 0.13-0.17.

The term "non-physiological conditions" means a pH which is not a physiological pH, either below or above pH 5-7.5 and ionic strength below and above 0.13-0.17.

The term "biological fluid samples" in this connection means blood, serum, urine, milk samples, etc., but also include homogenized tissue samples as well as standard solutions containing carrier proteins, such as albumin.

The term "proteinaceous substances" means proteins, such as immunoglobulins, antibodies, enzymes, protein hormones and carrier proteins, such as albumin, which are frequently present in biological samples.

The term "capturing" means that a substance in a biological fluid is capable of being bound to a "solid carrier".

The term "captured substance" means a substance, which can be bound on the environmentally sensitive solid carrier from the biological fluid. The binding is not necessarily due to the charges carried by the captured substance. Also substances, such as drugs, drug-like substances and drug-derivatives, which have an overall charge, which is neutral but carries residues, which can be charged or have a great affinity for the environmentally sensitive capturing residues, especially for poly(acrylic acid) on the inert solid carrier are included in this definition. The term "captured substance", however, does not include substances with the same charge as the "capturing residue".

The term "charged non-proteinaceous substances" include chemical compounds being or having residues, which either are positively or negatively charged in physiological conditions or they are neutrally charged but react to changes in the environment or the physiological conditions by being bound or released respectively. Such substances include chemoterapeutic substances, pharmaceutical products, drugs, drug derivatives,

steroids, non-proteinaceous hormones, such as steroid hormones, transmitter substances, such as serotonine, dopamine, gamma-aminobutyric acid (GABA), acetylcholine, etc., vitamines, provitamines, natural health food products, nucleic acids, including DNA, RNA and components and fragments thereof.

By the term "drugs" is meant relatively small chemical substances, including prodrugs, i.e. pharmaceutically active substances, which act as medicals as well as health food products, but also substances acting as poisons or narcotics.

The term "drug-like substances" is used in the present invention to include especially steroid hormones, transmitters, prodrugs, etc., i.e. substances with somewhat larger molecular weight than the substances defined as "drugs".

By the term "drug derivatives" is meant any metabolites of the above defined as "captured substances" or "non-proteinaceous substances, drugs, drug-like substances including both their degraded, conjugated or otherwise modified products.

The term in "inert form" means that the "captured substances" or "non-proteinaceous substances", including drugs, drug-like substances and drug derivatives as well as nucleic acid are not essentially changed during the separation, isolation and purification procedure. The "inert polymer backbone" of the solid carrier does not participate actively in any of the processes. It only is a means for transporting the residues and captured substances in the method of the present invention.

The term "washing step" means rinsing the solid carrier with a suitable solvent or solution, e.g. aqueous solution or a buffer, etc. or incubating or dipping it in a suitable solvent or solution to remove loosely attached proteinaceous

materials.

The term "releasing" means that the "captured substances", which are captured or bound from the biological fluid sample onto the solid carrier are released in a solution, which is either different from or the same as the washing solution.

The term "contacting" means that the environmentally sensitive "capturing residues" on the solid carrier are allowed to react with or bind to the substances in the biological fluid sample.

The term "releasing solution" means a solution the pH of which is or can be changed to a non-physiological pH. The "releasing solution" is compatible with or used as a solvent in the monitoring or evaluation system used, or alternatively, it is acceptable as a liquid pharmaceutical carrier.

The term "test kit" means one or more solid carriers and/or auxiliary equipments, diluents and solvents in a packaged combination which is manufactured and sold with appropriate instructions for use.

The General Description of the Invention

During studies related to drug transport across porous ion exchange membranes, the present inventors surprisingly discovered that drugs did not move across polyacrylic acid grafted membranes as fast as expected due to the binding on the membrane surface (Åkerman, S., et al., Int. J. Pharm., 164 (1-2), 29-36 (1998)). Based on this finding the methods and solid carriers of the present invention were developed and their efficiency was evaluated. It was found that poly(acrylic acid) grafted polymers were especially effective in binding charged or non-charged drug molecules from body fluids under physiological conditions, while proteinaceous substances normally present in body fluids were efficiently excluded. The release of the isolated drugs was easy to perform by changing

the environmental conditions, i.e. pH or ionic strength of the liquid solvent, which is compatible with or used in the monitoring or evaluation method or as a pharmaceutically active liquid carrier. The effect of ionic strength on the poly(acrylic acid) membrane studied by Åkerman, S., et al., Pharm. Res., 15 (5), 802-805, (1998).

The present invention was developed based on these original findings and is directed to a simple but efficient method for capturing from biological fluid samples, non-proteinaceous substances, such as drugs, drug derivatives, drug-like substances, nucleic acid, etc. by binding said substances to a solid carrier under physiological conditions and releasing said substances by using a liquid solvent, the pH of which is or can be change to a non-physiological pH and which solvent is compatible with or used in the monitoring or evaluation system or acceptable as a liquid pharmaceutical carrier. The greatest advantage of the method is the effective removal of proteinaceous materials, which disturb the subsequent monitoring and evaluation system and diminish the specificity of the subsequent determination systems, especially the specificity of immunoassays.

Based upon these basic findings the preferred solid carriers of the present invention were developed. Said solid carriers for isolation, separation and purification are prepared of porous, inert polymeric material, the surface of which is provided with a substance, preferably a polymeric substance carrying a multitude functional groups, which have charges, which are opposite to those of the substance(s) to be monitored in physiological conditions and binds essentially no such proteinaceous substances, which most frequently are present in biological samples. The solid carrier is provided in the form of membranes, sheets, sticks, test tubes, plates or microplates as well as granules and beads.

The invention also discloses solid carriers made of porous, inert materials in the form of membranes, sheets, sticks, plates, test tubes or microplates as well as granules or beads attached to further solid supports. It is essential that the surface of the solid carriers can be provided with residues, which are charged at physiological pH-ranges and capable of capturing from biological fluid samples oppositely charged substances, but essentially no proteinaceous components normally present in biological fluids.

The most preferred embodiment of the present invention relates to poly(acrylic acid) polymers and their ability to bind drug molecules. The poly(acrylic acid) may be grafted on porous polymer backbones or polymer granules. Drug molecules can be bound to the polymer from the biological fluids (plasma, serum, urea) in physiological conditions. Therefore, this invention is assumed to be significant in clinical drug monitoring, especially in situations where the therapeutic drug concentration is to be followed up during medication.

In the most preferred embodiment of the invention poly(acrylic acid) or poly(vinyl pyridine) is grafted on a porous polymer backbone or scaffold. An example of such polymers is e.g. poly(acrylic acid) grafted poly(vinylidene fluoride) membranes, which are porous polymeric membranes, which are sensitive to environmental changes. In physiological conditions, the poly(acrylic acid) chains on the membrane are dissociated and the membrane is negatively charged. At physiological conditions the poly(acrylic acid) grafted poly(vinylidene fluoride) membrane is capable of binding drug or drug-like molecules or drug-derivatives with an positive charge, an overall neutral charge but with residues having at least a momentary positive charge due to environmental conditions. When environmental conditions, such as pH is decreased, the poly(acrylic acid) chains becomes undissociated and neutral at charge and consequently the bound drugs,

drug-like substances or molecules as well as drug-derivatives leave the solid carrier or membrane. These changes in the conformational state of the poly(acrylic acid) chain and in the charge of the solid carrier or membrane are reversible. Because the fluids of the body have a pH of about 7 and many drug molecules are positively charged or neutral in these conditions, it is possible to use poly(acrylic acid) grafted poly(vinylidene fluoride) membranes for isolation and purification of drugs from the body fluids. The use of poly(acrylic acid) grafted poly(vinylidene fluoride) membrane is especially suitable for the isolation of drugs and makes the analysis more simple and time resuming and allows novel methods to be developed for monitoring, analytical chromatography and immunoassays, especially for the analyses of steroid hormones and transmitter substances.

For substances having under physiological conditions an overall negative charge, the solid carrier should be covered with positive charges. The first such membrane to be tested was a vinylpyridine grafted poly(vinylidene fluoride) membrane, which was able to bind nucleic acids. Miller, I.R. has in Biochimica et Biophysica Acta 103, 219-230, 1965 described that there is an interaction between microparticles manufactured by vinylpyridine and DNA. The strength of the interaction is dependent of the quaternary nitrogen atoms. If grafted on the inert polymer backbone the quaternary nitrogen atoms of vinylpyridine are free and such solid carriers capable of binding nucleic acids, including DNA, RNA, cDNA, mRNA, etc. as well as fragments thereof, but not proteins from biological samples.

The method of the present invention comprises the steps of contacting a biological fluid sample under physiological conditions with a solid carrier made of a porous, inert polymeric material, the surface of which is covered with a substance, preferably a polymeric substance carrying a multitude of functional groups, which have charges, which are

opposite to those of the substance(s) to be monitored and binds essentially none of the proteinaceous substances which are most frequently present in biological samples.

The present invention is also related to a method for isolation, separation and/or purification from a biological fluid sample of charged non-proteinaceous substances to be monitored, such as drugs, drug derivatives or drug-like compounds or nucleic acids.

The carrier is allowed to be in contact with the biological sample for a time sufficient for the binding to occur. Optionally, the biological sample is incubated and gently stirred or the carrier is gently moved in the sample in order to facilitate an improved binding.

After the binding is completed the solid carrier is removed from the biological fluid sample and subjected to one or more optional washing steps. Alternatively, the solid carrier is directly contacted with a liquid solution the pH of which is below or over physiological pH or the pH of the solution is changed so that it is higher or lower than the physiological pH after the carrier has been inserted into the solution.

The conformation of the poly(acrylic acid) chains of the membrane is greatly dependent on the ionic strength and pH. When ionic strength is increased, the carboxylic acid chains of the membrane become more compact. In dilute solutions carboxylic acid chains are expanded. Also releasing rate of the drug molecules from the PVDF-PAA membrane is greatly dependent of ionic strength of the surrounding buffer (Fig 6).

The time required for binding the charged substances in the biological fluid sample should be as short as possible, preferably not more than 1-2 hours, most preferably only a few minutes. However, the time can and should be adjusted so that it is sufficiently long to allow also substances

present in small amounts to be bound.

Positively charged bound substances, such as drugs are released by contacting them with a solution, the pH of which is lower than physiological pH or which is lowered by acidification with a suitable acidic solvent or buffer solution. The preferred non-physiological acidic pH is below 5, more preferably below 4, most preferably a pH of 2-3.

Negatively charged bound substances are released by contacting them with said monitoring solution having a pH higher than the physiological pH. The preferred non-physiological basic pH is a pH over 7.5, more preferably above than pH 8, most preferably a pH in the range pH 8.5-9.

Further, there are the charged non-proteinaceous substances of the present invention, which react as a response to changes in ionic strength.

Interactions between several drugs in general therapeutic use and the grafted poly(acrylic acid) grafted polyvinylidene fluoride-membranes have been studied and it has been found that the drugs are bound to the membrane. Because the membrane does not bind other serum components such as proteins it is applicable as a matrix for selective separation of drugs. The membrane facilitates a simple and rapid drug separation. In addition, metabolites of the drug can be bound simultaneously. In order to apply the benefits of the membrane in separation processes more effectively, some practical modifications are schematically shown in Fig. 2 and Fig. 3.

Fig. 2 shows the method performed on a membrane. In step (1) the test tube containing a serum, plasma, or urine sample containing the drug and proteins is shown. In step (2) the same test tube is shown incubating the PVDF-PAA membrane in the sample. In step (3) the binding of drugs to the membrane

are shown. In step (4) the membrane is washed with distilled water. In step (5) the drugs are released in acid methanol and in step (6) the sample in the test tube is ready for analysis.

Fig. 3 shows the method according to the invention performed on the bottom of a test tube, which has been covered with grafted granules or beads. In step (1) the serum, plasma, or urine sample containing the drug and proteins are added to a test tube, the bottom of which is covered with solid carriers capable of capturing the drugs and incubated. In step (2) the drugs are bound to the bottom of the test tube. In step (3) the sample is removed. The step (4) the test tube is washed with distilled water. In step (5) the water is removed from the test tube. In step (6) the drugs are released in acid methanol and the sample in the test tube is ready for analysis.

Similar application can be provided on microplates, sticks, etc.

The present invention not only provides improved and simplified isolation, separation and purification procedures. It also improves the efficiency of the subsequent monitoring and evaluation procedures and can find several analytical applications as described below.

Chromatographic monitoring and evaluation

The present invention can be applied not only to isolation, separation and purification. It also improves the efficiency of the subsequent monitoring and evaluation procedures. For, example, the membrane based purification is integrated as an on-line system to HPLC equipments, the chromatography step is even more rapid to perform because the disturbing solvent front in HPLC-chromatography is decreased as shown in Fig. 5.

If the membrane based purification is integrated as an on-line

system to HPLC equipments, the chromatography step can be performed more rapidly. The disturbing solvent front in HPLC is decreased. This allows the chromatographic procedure to be performed in less time.

Immunochemical determinations

The present invention provides improved immunological methods, in which the non-specific binding can be significantly reduced. The solid carriers of the present invention, especially polyacrylic acid grafted carriers bind drugs. Thus, they can be applied in immunochemical drug determinations by using them as drug binding matrices. Such a matrix can bind several drugs, which can be simultaneously detected by using antibodies specifically binding to said drugs. Because the solid carrier of the present invention does not bind proteins, unspecific binding is significantly reduced. The proteins do not disturb the immunochemical reaction, which usually hampers the presently used immunological methods and usually prevents the simultaneous determination of several drugs from one sample.

In immunological methods the non-specific binding can also be significantly reduced. The solid carriers of the present invention, especially polyacrylic acid grafted carriers bind drugs. Thus, they can be applied in immunochemical drug determinations by using them as drug binding matrices. Such a matrix can bind several drugs, which can be simultaneously detected by using antibodies specifically detecting said drugs. Because the solid carrier of the present invention does not bind proteins, unspecific binding can be significantly reduced and thus it does not disturb the reaction, which hampers the presently used immunological methods and usually prevents the simultaneous determination of several drugs.

Determination of drugs from patient derived samples

Methods for determining drugs in different samples from patients are required especially when the treatment is started with a drug with a narrow therapeutic window (applicability). Nowadays, the concentration of drugs are often determined during a prolonged time in order to follow up the success of medication.

In addition, before starting the rapidly required treatment in an effective way, the patient presenting with poisoning indicating symptoms, a rapid and simple analyses of the cause of poisoning is required.

Test kits for separate, specific determinations can be provided in packaged combinations of the solid carrier, including tubes, membranes, microplates, etc. or beads or granules with appropriate instructions.

A further application of the present invention is related to steroid hormones and transmitters, such as serotonine, dopamine, gamma-aminobutyric acid, acetylcholine, etc.

The method, system and solid carrier according the present invention as well as their use are described in more detail in the following example. The example should not be understood as limiting the invention. The skilled artisan can based on this description develop a multitude of similar solid carriers for drug separation and monitoring.

Example 1

(a) Preparation of poly(acrylic acid) grafted polyvinylidene fluoride sheets by preirradiation grafting

Hydrophobic polyvinylidene fluoride (PVDF) membranes, (Millipore) with pore sizes 0.22 μm, and acrylic acid (AA), (Aldrich, Steinheim, Germany) stabilized with 200 ppm hydroquinone, were used as received. Ion-exchange water was

used throughout.

Preirradiation grafting was accomplished by first irradiating the PVDF membranes under nitrogen atmosphere (<200 ppm O2) using Electrocurtain electron accelerator (Energy Sciences Inc.) operating at an acceleration voltage of 175 kV. The membranes were irradiated with 25 kGy. Immediately, after irradiation the membranes were immersed at ambient temperature in a graft solution containing AA. This solution was continuously purged with nitrogen in order to remove oxygen.

After grafting the membranes were soxhlet extracted with water to remove remaining monomer and dried in vacuo at 40 °C overnight.

(b) Determination of degree of drafting

The degree of grafting (G) was determined gravimetrically according to the formula

$$G = ((m1/m0)/m0)\ 100\ wt\%$$

where m0 represents the mass of the original PVDF membrane and m1 represents the mass of the grafted, extracted and dried PVDF membrane.

The reaction mechanism is depicted in Figure 1.

COMPARATIVE TESTS FOR EVALUATION OF THE EFFICIENCY

(a) Tested Drugs

Citolapram and desmethylcitolapram were purchased from Lundbeck (Copenhagen, Denmark). Clomipramine and norclomipramine were from Ciba-Geigy (Basel, Switzerland) Clozapine and norclozapine were from Sandoz (Basel, Switzerland). Fluoxetine and norfuoxetine were from Lundbeck

(Copenhagen, Denmark). Acetonitrile and methanol (HPLC grade) as well as other reagents (analytical grade) were obtained from Merck (Darmstadt, Germany).

(b) Preparation of test solutions

The stock solutions of drugs were made by dissolving 10 mg of drug in 10 mL methanol. Finally the test solutions were prepared to 50 g/L albumin solution (ionic strength 0.15 mol/l with NaCl) as follows. The appropriate volumes of stock solutions were evaporated to dryness at 50 °C with a gently stream of air and the residue was dissolved in 100 mL of albumin solution by mixing it gently with a magnetic stirrer at room temperature for 30 min. Serum samples were made by diluting the stock solutions of drugs to a appropriate concentration with pooled drug free serum. Citalopram (300 nM), desmethylcitalopram (300nM), clomipramine (900 nM), norclomipramine(900 nM), clozapine (4500nM), norclozapine (4500nM), fluoxetine (900nM) and norfluoxetine (900nM) were used in studies. The drug concentrations were correspondent to the high therapeutic ones in serum of the patients.

(c) Sample preparation with solid phase extraction

The samples were prepared with an ASPEC automatic sample preparator (Gilson Medical Electronics, Villiers-le-Bel, France) using 100 mg Bond-Elut C-18 solid-phase extraction columns (Varian, Sunnyvale, CA, USA). The extraction column was activated with 2.0 mL of methanol and washed with 2.0 mL of HPLC grade water. The whole sample was added to the column followed with two washing steps of 2.0 mL HPLC grade water. The column pH was set as acidic with 1.0 mL of 10% of methanol in 0.25 M hydrochlorid acid. Before the elution step column was washed with 500 µL of acetonitrile. The analytes were eluted with two 500 µL portions of 10 mM acetic acid, 5 mM diethylamine in methanol. Finally the eluates were evaporated to dryness with Techne Sample Concentrator (Techne, Cambridge,

UK) in 37C with a gentle stream of air. The residue was reconstituted in 100 L of mobile phase. The injection volume was 40 µl.

(d) Instrumentation

The HPLC system consisted of Hewlett Packard 1020 liquid-chromatography controlled by Chemstation chromatography controlled by ChemStation chromatography workstation (Hewlett Packard, USA). Chromatographic separations were achieved using a Select-B (4 µm, 125 x 4 mm) C-18 analytical column (Merck, Darmstadt, Germany). The elution was isocratic with a mobile phase of acetonitrile: 50 mM dipotassiumhydrogen phosphate, pH 4.7 (40:100) at a flow rate of 1.2 mL/min. Drugs were detected at 220 nm and the peak purity analyses were performed at 210-365 nm.

(e) Isolation of drugs with polyacrylic acid grafted poly(vinylidene fluoride) membrane

Various grafted poly(vinylidene fluoride) membranes were incubated in the drug solution over night in horizontal shaker at room temperature. After incubation, membranes were washed with distilled water and chelated drugs were released to acidic methanol (pH (3). Methanol was evaporated to dryness with Techne Sample Concentrator (Techne, Cambridge, UK) in 37 °C with a gentle stream of air. The residue was reconstituted in 100 L of mobile phase and drug concentrations were determined with HPLC. The injection volume was 40 µl.

1. Binding of serum components to the PVDF-PAA -membrane

Pooled serum was divided into the 6 samples of 1 ml. The membranes with pore size 0.22 µm and a diameter of 9 mm were incubated in horizontal shaker over night in the three of the serum samples to evaluate the components, which are binding to the membrane. Other three serum samples were used as controls.

After incubation membranes were washed with 1 ml of distilled water, 1ml of 10 mM phosphate buffer pH 3.7 and 1 ml of acidic methanol (pH 3), respectively. Methanol was evaporated to dryness as described above. The residues were reconstituted in 1 ml of 10mM phosphate buffer pH 3.7. The absorbed serum components were measured from control samples, incubation solutions (Table 1) and wash solutions of the membranes and soluted methanol residues. From the wash solutions and soluted methanol residues the concentrations of serum components were not detectable. The serum Ca bound to the membrane very effectively, but from the membrane it was neither released to the wash solution nor to the methanol used as a releasing solution.

Table 1. The measured amount of the serum components (%) in the incubation solution after the treatment with the membrane

The component of the serum	The amount of the serum component (%) after membrane treatment
Immunoglobulin A	97.59
Immunoglobulin G	96.70
Immunoglobulin M	125.15
Albumin	96.04
Transferrin	94.09
Alkaline phosphatase	99.48
Aspartate aminotransferase	105.78
Alanine aminotransferase	101.70
Amylase	96.52
Creatine kinase	103.45
Glutamyltransferase	102.94
Lactate dehydrogenase	104.20
Total protein	103.24
Albumin	104.04
Creatinine	108.10
Urea	103.87
Urate	102.48
Cholesterol	104.77
Triglycerides	101.53
Bilirubin	103.85
Fe	108.26
Ca	15.70
Ca ionized	7.31
Na	99.95
K	99.76

2. Effect of the degree of grafting to the drug binding

10 wt% and 19 wt%, grafted membranes were used to study the effect of degree of grafting on the drug binding. Results are shown in Table 2.

Table 2. Effect of degree of grafting on the drug binding on the membrane.

Drug	Recovery* (10 wt% grafted membrane	Recovery* (19 wt% grafted membrane)
Norclomipramine	63.47	73.58
Clomipramine	68.69	71.68
Norcitalopram	52.24	55.85
Citalopram	46.52	70.66
Norclozapine	54.50	52.48
Clozapine	59.12	55.86
Norfluoxetine	61.64	49.68
Fluoxetine	64.24	58.38

* Recovery as % from recovery of C-18 extracted samples.

3. Binding of drug molecules from serum to the PVDF-PAA -membrane

The binding of 51 drug molecules in the 19 wt% grafted membrane were tested. The used drug molecules, their concentrations and binding percents are shown in Table 3.

Table 3. The binding of 51 drug molecules in the 19 wt% grafted membrane.

Drug	mw	logP	pKa	Bound to the membrane (%)
Primidone	218.26	0.91;1.74	-	1.16
Lamotrigine	256.16	2.08	-	37.41
Phenobarbital	232.24	1.47;1.36	7.4	2.74
Carbamazepine	236.28	2.45;1.98	-	28.72
Phenytoin	252.28	2.47;2.09	8.3	5.68
Pentobarbital	226.28	2.07;2.11	8.0	5.70
Nitrazepam	281.27	2.25;2.53	3.2;10.8	41.4
Clonazepam/ Alprazolam	308.77	3.20	-	81.65
Flunitrazepam	313.29	2.06;2.36	1.8	93.65
Medazepam	270.76	4.41;4.47	6.2	77.46
Monohydroxy-carbazepine	-	-	-	8.89
Oxcarbazepine	252.28	1.21	-	17.37
Zopiclone	388.82	0.98	-	29.91
Nordiazepam	270.72	2.93;3.16	3.5;12.0	49.54
Diazepam	284.75	2.8;3.18	3.3	91.26
Midazolam	325.78	3.7	6.2	101.26
Oxazepam	286.72	2.24;2.1	1.7;11.6	16.80
Norclobazam	-	-	-	20.62
Temazepam	300.75	2.19;2.4	1.6	39.64
Clobazam	300.75	2.65;0.95	-	57.61
Nordoxepine	-	-	-	53.70
Doxepine	279.39	3.88	9.0	44.63
Maprotiline	277.41	4.22	-	43.38
Normaprotiline	-	-	-	62.45
Nortrimipramine	-	-	-	61.05
Trimipramine	294.44	4.73	-	52.13
Clorprotixene	315.87	5.18;5.30	7.6	52.16
Thioridazine	370.58	5.90;6.42	9.5	27.20
Trazodone	371.87	4.0	-	37.29
Haloperidole	375.87	3.36;3.52	8.3	75.01
Nortriptyline	-	-	-	58.66
Amitriptyline	277.41	5.04;4.64	9.42	61.35
Thiotixene	443.63	3.78;4.80	7.67;7.97	16.59
Norclozapine	-	-	-	28.86
Clozapine	326.83	3.62;2.99	8.0	29.54
Desipramine	266.39	4.9;4.09	10.44	59.71
Imipramine	280.42	4.80;4.41	9.5	52.99
Levomepromazine	-	-	-	38.97
Norclomipramine	-	-	-	59.90
Clomipramine	314.86	5.19;5.30	9.38	69.65
Norcitalopram	-	-	-	74.36
Citalopram	324.4	2.98	-	47.78
Mianserine	264.37	4.26	-	51.36
Protriptyline	263.39	4.32	-	82.20
Norfluoxetine	-	-	-	53.66
Fluoxetine	309.33	4.05	-	86.37
Chlorpromazine	318.87	5.35;5.20	9.30	49.24

4. Effect of incubation volume of serum to the binding of drugs from serum to the PVDF-PAA -membrane

The effect of incubation volume of serum were tested using volumes of 2000 µl, 1000 µl, 500 µl, 250 µl and 100 µl. Results are shown in Table 4.

Table 4. Effect of incubation volume of the serum sample on recovery with 19wt% grafted membrane.

Volume of serum sample (µl)	Recovery (%)*
Norclomipramine	
100	65.21
250	77.19
500	39.55
1000	62.30
2000	37.48
Clomipramine	
100	81.63
250	98.86
500	51.62
1000	76.09
2000	44.21
Norcitalopram	
100	74.14
250	98.98
500	75.00
1000	104.77
2000	99.30
Citalopram	
100	47.77
250	87.05
500	62.64
1000	89.33
2000	87.23
Norclozapine	
100	87.52
250	76.70
500	nd
1000	96.48
2000	48.98
Clozapine	
100	85.30
250	83.37
500	nd
1000	98.9

(cont.)

Volume of serum sample (µl)	Recovery (%)*
2000	44.65
Norfluoxetine	
100	153.89
250	152.45
500	nd
1000	138.91
2000	104.40
Fluoxetine	
100	107.71
250	103.33
500	nd
1000	94.40
2000	44.56

* Recovery as % from C-18 extracted serum samples

5. Effect of incubation time to the binding of drugs from serum to the PVDF-PAA membrane

The incubation times of 30 min, 90 min, 180 min 360 min and 24 h were used to study the effect of incubation time on drug binding. Results are shown in Table 5.

Table 5. The effect of incubation time to the recovery of 19wt% grafted membrane

Incubation time (min)	Recovery (%)
Norclomipramine	
30	12.99
90	16.79
180	49.06
360	54.43
over night	62.30
Clomipramine	
30	11.35
90	25.48
180	44.48
360	50.13
over night	76.09
Norcitalopram	
30	34.84
90	61.80

(cont.)

Incubation time (min)	Recovery (%)
180	91.33
360	85.39
over night	104.77
Citalopram	
30	24.67
90	50.92
180	76.64
360	70.08
over night	89.33
Norclozapine	
30	10.10
90	28.71
180	47.75
360	80.89
over night	96.48
Clozapine	
30	8.40
90	25.71
180	40.06
360	70.11
over night	98.90
Norfluoxetine	
30	25.38
90	61.09
180	58.35
360	66.07
over night	138.91
Fluoxetine	
30	47.84
90	58.03
180	70.96
360	126.29
over night	94.40

* Recovery % from C-18 extracted samples

6. Effect of the methanol volume on the drug release from PVDF-PAA -membrane

The volumes of 100 µl, 250 µl, 500 µl and 1000 µl were used to release the bound drugs from the membrane. Results are shown in Table 6.

Table 6. Effect of the methanol volume on the drug release from the 19 wt% grafted membrane

Volume of methanol (µl)	Recovery (%)*
Norclomipramine	
100	23.76
250	48.54
500	78.26
1000	61.65
Clomipramine	
100	37.20
250	72.05
500	81.88
1000	80.14
Norcitalopram	
100	23.58
250	58.46
500	65.78
1000	78.30
Citalopram	
100	40.77
250	82.77
500	91.04
1000	93.55
Norclozapine	
100	49.69
250	80.11
500	88.64
1000	84.76
Clozapine	
100	63.36
250	74.95
500	84.77
1000	83.25
Norfluoxetine	
100	30.01
250	57.62
500	63.14
1000	63.44
Fluoxetine	
100	28.42
250	63.17
500	62.59
1000	62.92

The binding of the drug on the membrane surface is most effective when the logP value of the drug is between 2 and 4. At very low and high logP values, the binding is at lower

level. However, recovery of the oxazepam is only 16.8 % even it has the pKa about 2. This might be due the acidic nature of the oxazepam.

When the grafting rate of the membrane is over 40 wt%, the drug molecules binds to the membrane surface. Grafting rate of about 10 wt% to 20 wt% seem to be most ideal in selective drug binding from the biological fluids when volume of the incubation sample is between 100 µl to 1 ml. There is no significant differences in recovery between 10 wt% and 19 wt% grafted membranes. If incubation volume of the sample is smaller and incubation time shorter, more grafted membrane might give better recovery for drug binding.

The area and the grafting rate of the exposed membrane is in the correlation to the volume of incubation sample. As seen in Table 4, area of used 19 wt% grafted membrane (0.36 cm) is ideal for the sample of 1 ml. Volume of 2 ml should require bigger membrane area for better recovery. At low sample volumes also the smaller membrane area should get good recoveries.

When short incubation time is used with sample volume of 1 ml, the recovery is lower. By optimization of the incubation time, incubation volume of the sample and membrane grafting rate, it is possible to develop efficient and timesaving method for drug binding from small amounts of biological fluids.

The bound drug was released from the membrane to the methanol. Methanol volume of 250 µl gave recovery of about 50 % to the all tested drugs (Table 6). If used area of the membrane is smaller, method gives possibility to filtrate the small methanol volume through the membrane to the HPLC column.

These preliminary evaluations indicates that the method of the invention works and even better results are obtainable by optimizing the parameters.

Ionic strength

The conformation of the poly(acrylic acid) chains of the membrane is greatly dependent on the ionic strength. When ionic strength is increased, the carboxylic acid chains of the membrane become more compact. In dilute solutions carboxylic acid chains are expanded. Also releasing rate of the drug molecules from the PVDF-PAA membrane is greatly dependent of ionic strength of the surrounding buffer (Fig 6). Drug is released more slowly at low ionic strength. When ionic strength of the buffer is increased, drug releases in a more rapid manner.

Example 2

Vinylpyridine grafted poly(vinylidene fluoride)

(a) Preparation

Hydrophobic polyvinylidene fluoride (PVDF) membranes, (Millipore) with pore sizes 0.22 μm, and polyvinylpyridine, stabilized with 200 ppm hydroquinone, were used as received. Ion-exchange water was used throughout.

Preirradiation grafting was accomplished by first irradiating the PVDF membranes under nitrogen atmosphere (<200 ppm O2) using Electrocurtain electron accelerator (Energy Sciences Inc.) operating at an acceleration voltage of 175 kV. The membranes were irradiated with 25 kGy. Immediately, after irradiation the membranes were immersed at ambient temperature in a graft solution containing AA. This solution was continuously purged with nitrogen in order to remove oxygen.

After grafting the membranes were soxhlet extracted with water to remove remaining monomer and dried in vacuo at 40 °C overnight. The grafting was performed essentially as described

in Example 1.

(b) Determination of degree of grafting

The degree of grafting was determined essentially as described in example 2.

ISOLATION OF NUCLEIC ACIDS

25 wt% grafted VP-PVDF -membrane was incubated in digested HS-DNA solution (10 mg/ml Tris-EDTA) (Sigma) at pH 7.5 at room temperature over night. After incubation membrane was washed with distilled water and bound DNA was released to the 1 ml of methanol solution (pH 10 with NaOH). Released amount of DNA was determined spectrophotometrically at 260 nm. 30 ng of DNA was released from the membrane after incubation. Membrane area exposed was 0.36 cm^2. Blank test was made by incubating 25 wt% grafted membrane in Tris-EDTA buffer pH 7.5, which contained no DNA.

What we claim:

1. A method for separating non-proteinaceous substances, such as drugs and/or nucleic acids from proteinaceous substances in biological fluid samples for subsequent monitoring and evaluation, c h a r a c t e r i z e d in that it comprises the steps of:

(a) contacting said biological fluid sample under physiological conditions with at least one solid carrier, the surface of which is covered with a substance, preferably a polymer forming substance carrying a multitude of capturing residues, which are sensitive to environmental conditions and which under physiological conditions, are capable of capturing said non-proteinaceous substances but essentially none of the proteinaceous substances present in biological samples;

(b) removing said solid carrier carrying the captured substances captured by the capturing residues in step (a) and subjecting the solid carrier to one or more optional washing steps;

(c) releasing said captured substances by contacting the solid carrier with a releasing solution under non-physiological environmental conditions.

2. The method of claim 1, c h a r a c t e r i z e d in that the solid carrier is a porous, inert polymeric material manufactured in a form that facilitates easy separation, such as a membrane, sheet, stick, test tube, plate or microplate as well as beads or granules attached to a solid support.

3. The method of claims 1-2, c h a r a c t e r i z e d in that the environmentally sensitive capturing residues of the solid carrier are selected from a group consisting of acrylic acid, poly(acrylic acid), vinylpyridine, or poly(vinylpyridine).

4. The method of claims 1-2, c h a r a c t e r i z e d in that the solid carrier provided with capturing residues is poly(acrylic acid) grafted polyvinylidene fluoride.

5. The method of claims 1-4, c h a r a c t e r i z e d in that the environmentally conditions include pH and ionic strength.

6. The method of claim 5, c h a r a c t e r i z e d in that the releasing solution is compatible with or used as a solvent or diluent in the method for monitoring or evaluating the captured substance.

7. The use of a solid carrier for separating non-proteinaceous substances, such as drugs and/or nucleic acids from proteinaceous substances in biological fluid samples for subsequent monitoring and evaluation according to methods of claims 1-6, c h a r a c t e r i z e d in that the solid carrier is a porous, inert polymeric material in the form of membranes, sheets, sticks, test tubes, plates, microplates or as granules or beads attached to a further solid support, the surface of said solid carrier being covered with a substance, preferably a polymeric substance carrying a multitude of capturing residues, which are sensitive to environmental conditions and which under physiological conditions are capable of binding said non-proteinaceous substances but essentially none of the proteinaceous substances present in the biological samples.

8. The solid carrier of claim 7, c h a r a c t e r i z e d in that the environmentally sensitive capturing residues of the solid carrier are selected from a group consisting of acrylic acid, poly(acrylic acid), vinylpyridine, or polyvinylpyridine.

9. The solid carrier of claims 7-8,

c h a r a c t e r i z e d in that the solid carriers provided with capturing residues comprises poly(acrylic acid) grafted polyvinylidene fluoride.

10. A test kit for separation of non-proteinaceous substances such as drugs and/or nucleic acids from proteinaceous substances in a biological fluid sample for subsequent monitoring and evaluation, c h a r a c t e r i z e d in that it comprises one or more solid carriers of a porous, inert polymeric material, the surface of which is covered with a substance, preferably a polymeric substance carrying a multitude of capturing residues, which are sensitive to environmental conditions and which under physiological conditions are capable of capturing said non-proteinaceous substances but essentially none of the proteinaceous substances present in biological fluid samples, provided in a packaged combination with optional washing solutions and at least one releasing solution, which is compatible with or used as solvent or diluent in the subsequent method for monitoring or evaluating the captured substance as well as auxiliary equipments and instructions for use.

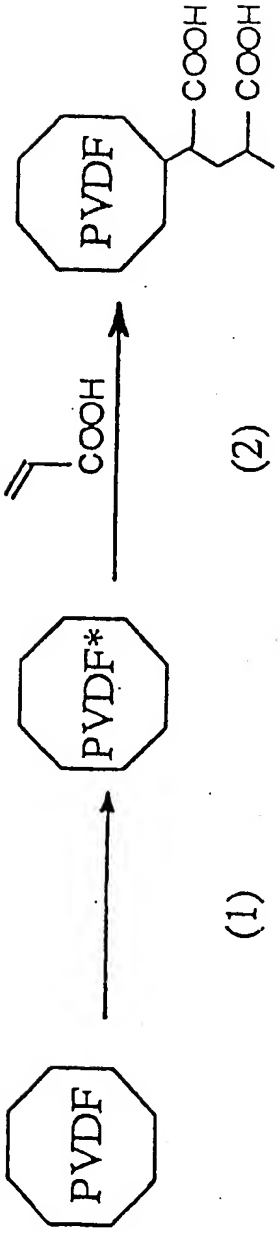


FIG 1

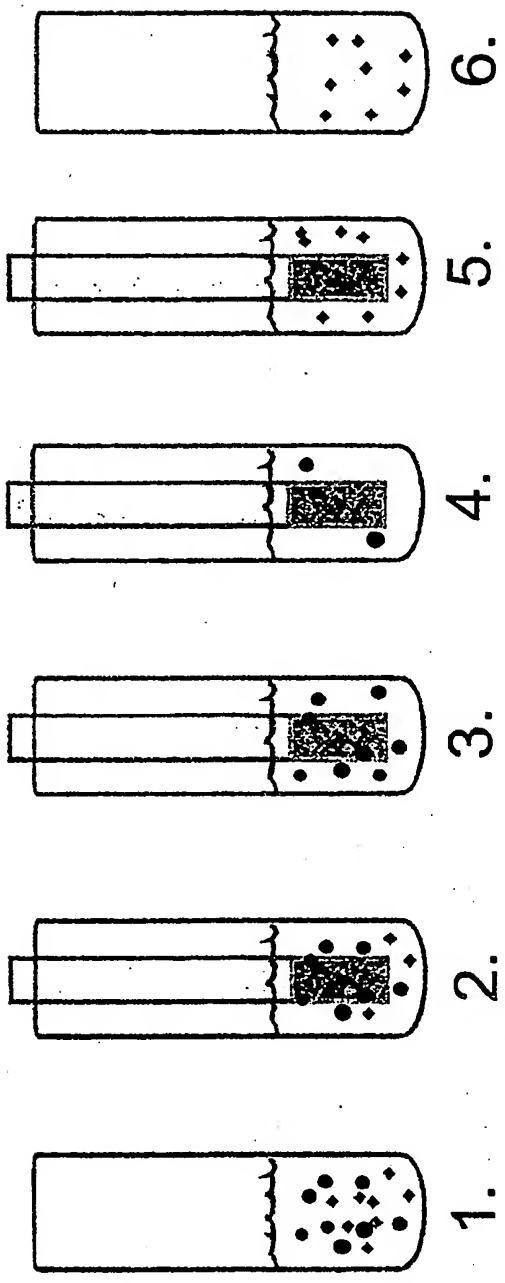


FIG 2

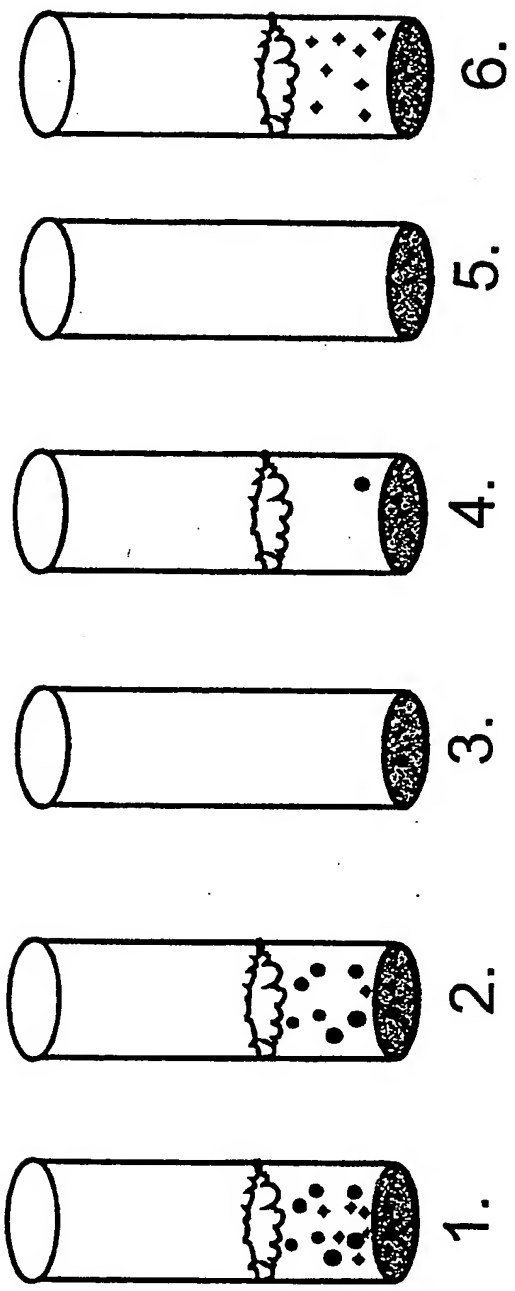
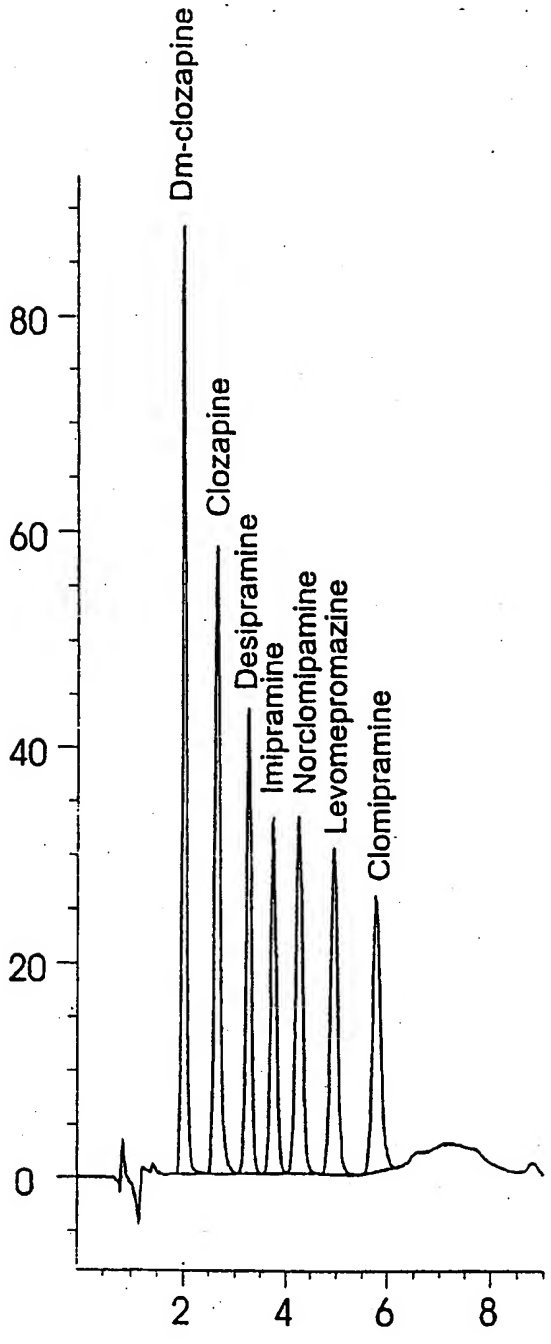


FIG 3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Clozapine
Desipramine
Imipramine
Norclomipamine
Levomepromazine
Clomipramine
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60
40
20
0
2
4
6
8

FIG 4A

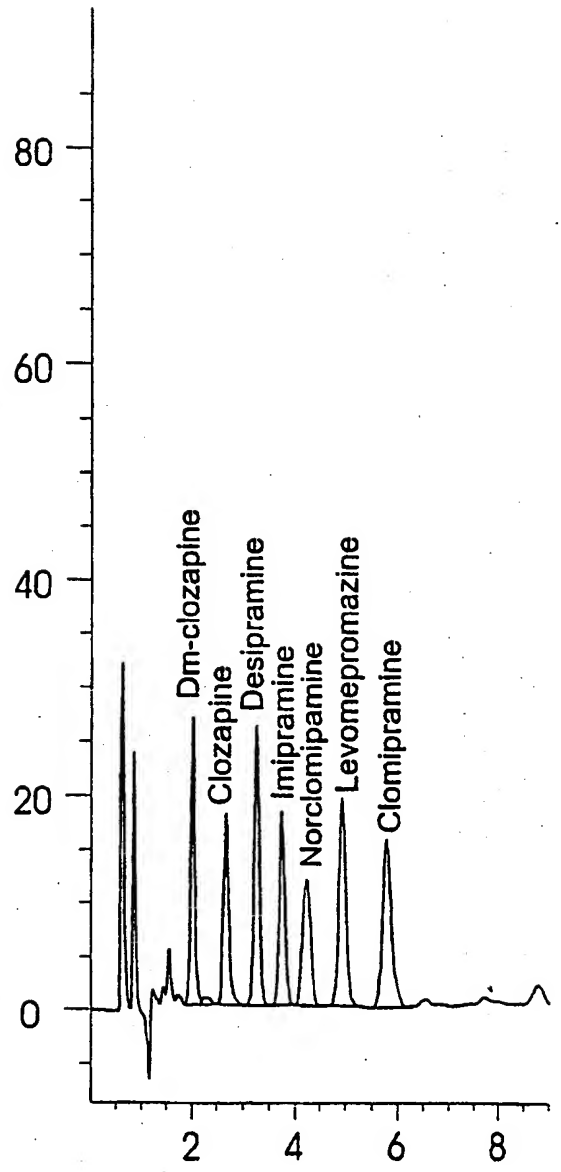
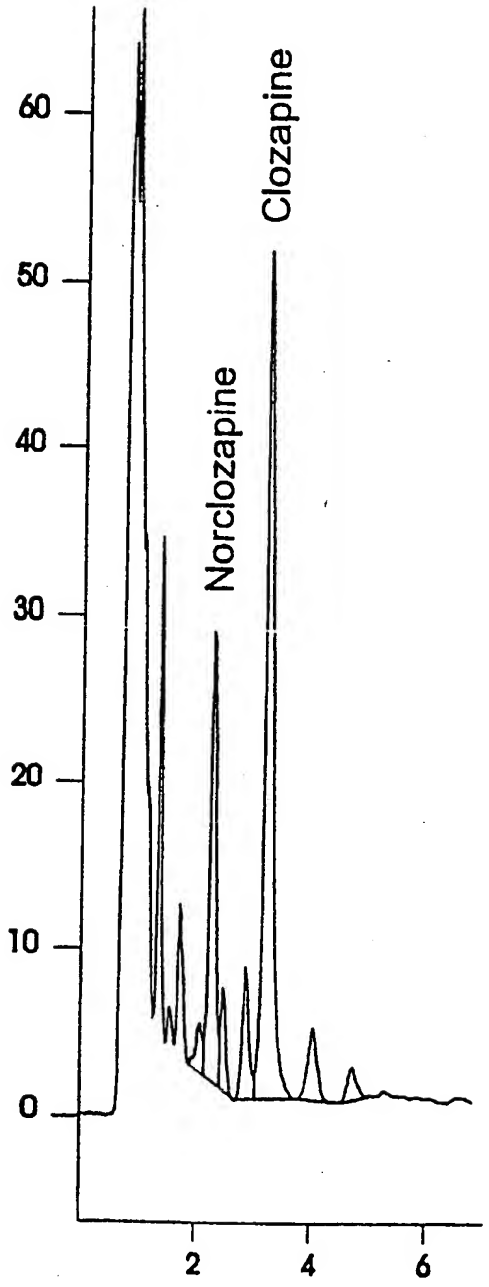

Dm-clozapine
Clozapine
Desipramine
Imipramine
Norclomipamine
Levomepromazine
Clomipramine
80
60
40
20
0
2
4
6
8

FIG 4B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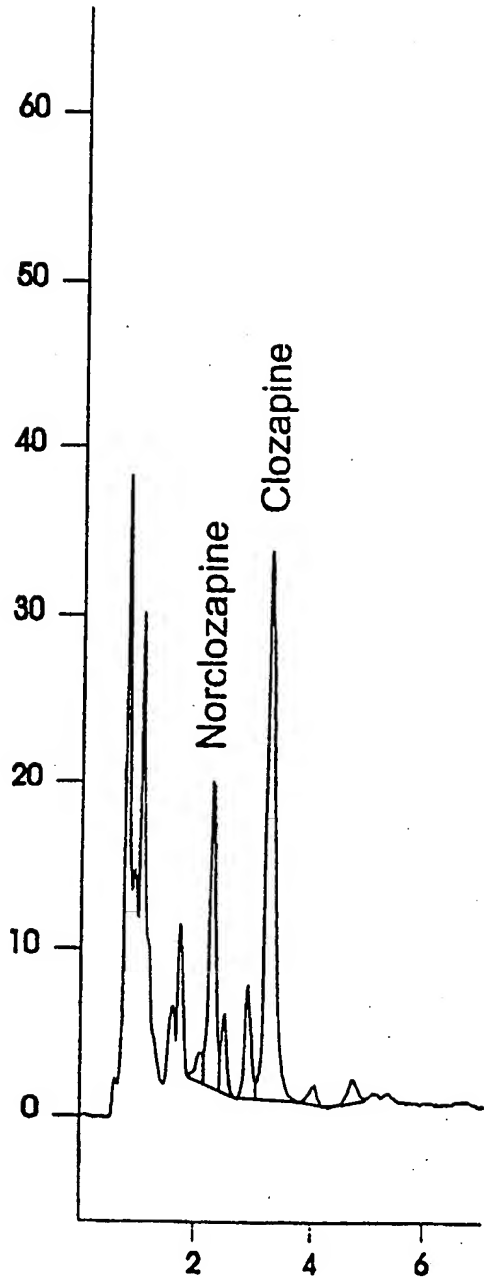
50
40
30
20
10
0
Norclozapine
Clozapine
2
4
6

FIG 5A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50
40
30
20
10
0
Norclozapine
Clozapine
2
4
6

FIG 5B

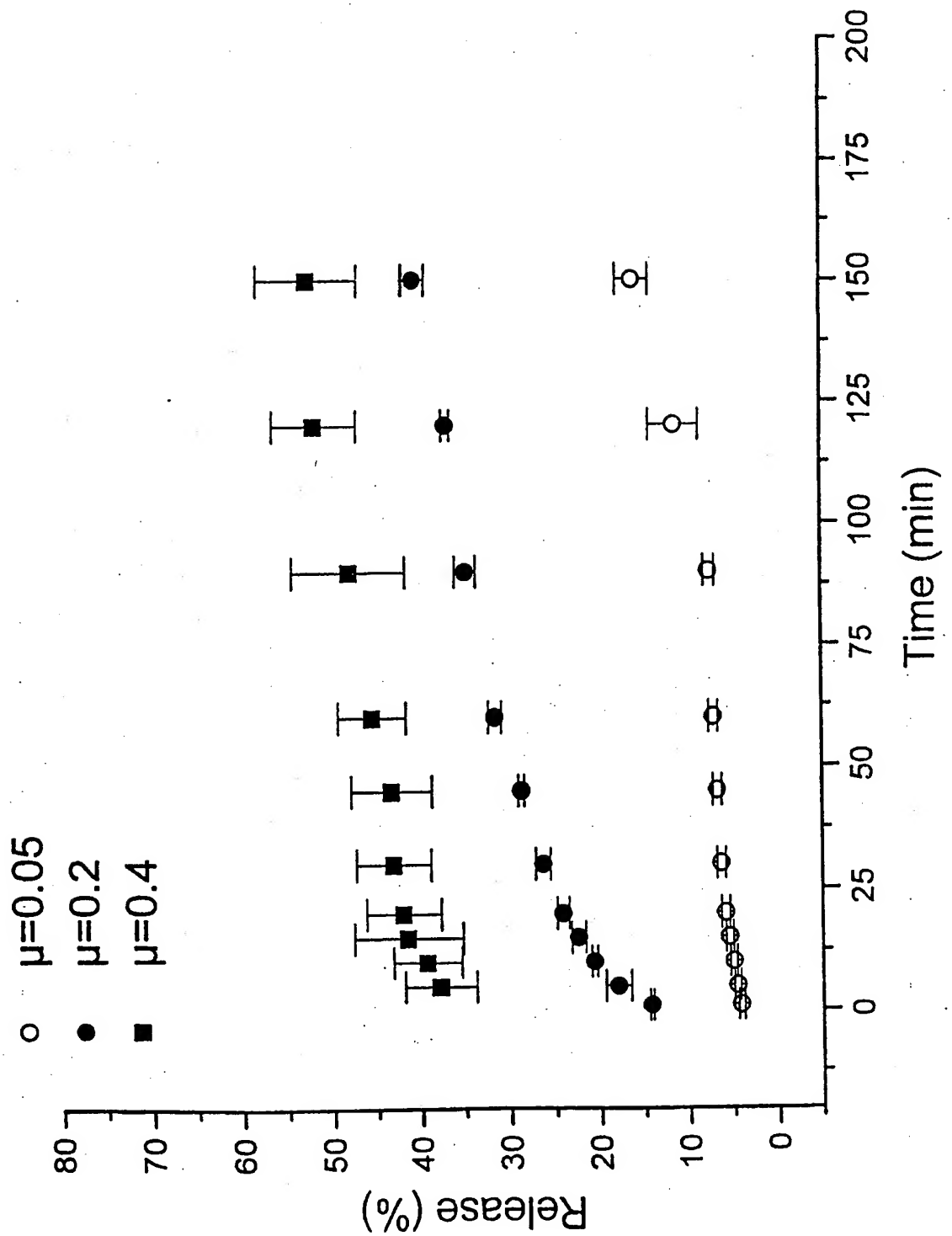
μ=0.05
μ=0.2
μ=0.4
Release (%)
Time (min)
0
10
20
30
40
50
60
70
80
0
25
50
75
100
125
150
175
200

FIG 6

INTERNATIONAL SEARCH REPORT

International application No.
PCT/FI 98/00852

A. CLASSIFICATION OF SUBJECT MATTER

IPC6: G01N 30/00, G01N 33/48
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPI, PATENT ABSTRACTS OF JAPAN, SCISEARCH

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 0268946 A2 (DIAGEN INSTITUT FÜR MOLEKULARBIOLOGISCHE DIAGNOSTIK GMBH), 1 June 1988 (01.06.88), column 6, line 25 - line 36; column 7, line 55 - column 8, line 23, figure 4	1,7,10
Y	--	2-6
X	Ind. Eng. Chem. Res., Volume 35, 1996, Joni Hautojärvi et al, "Characterization of Graft-Modified Porous Polymer Membranes", page 450 - page 457, see page 450, paragraphs 1 and 4	8-9
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[X] Further documents are listed in the continuation of Box C. [X] See patent family annex.

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17 February 1999	27 -02- 1999
Name and mailing address of the ISA/ Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. +46 8 666 02 86	Authorized officer Carl-Olof Gustafsson Telephone No. +46 8 782 25 00

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C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Macromolecules, Volume 17, 1984, Takahiro Seki et al, "pH-Sensitive Permeation of Ionic Fluorescent Probes from Nylon Capsule Membranes" page 1880 - page 1882	1-10
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INTERNATIONAL SEARCH REPORT

Information on patent family members

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